PRELIMINARY GENETIC ANALYSIS OF

SILENE SPALDINGII (Spalding's Catchfly),

A CANDIDATE THREATENED SPECIES

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EXECUTIVE SUMMARY

A genetic analysis of Silene spaldingii (Spalding's catchfly), a candidate threatened plant species, was initiated in 1994 to elucidate the distribution of genetic variation within and among populations. Enzyme electrophoresis was performed on 96 individuals from populations in Idaho, Montana, Oregon, and Washington. Of the seventeen enzymes screened for activity, six were resolved yielding four polymorphic and six monomorphic loci. A UPGMA cluster analysis of Nei (1978) unbiased genetic distance revealed a northern group consisting of the Dancing Prairie and Lamona populations, and a southern group consisting of the Kramer, Garden Creek and Clear Lake Ridge populations. Further, populations from Hells Canyon (ID & OR) are very similar (.000). A preliminary analysis of cpDNA (chloroplast) with restriction endonucleases indicates a length mutation in Open Reading Frame (ORF) 178 within the Lamona population, and possibly within the Dancing Prairie population, further uniting the southern populations. A Chi-squared analysis for deviation from Hardy-Weinberg equilibrium was used to test the hypothesis that outcrossing is the primary breeding system of Silene spaldingii. Results indicated that four of the five populations have frequencies of heterozygotes and homozygotes which would be expected as a result of random mating. The Dancing Prairie population contains an excess of homozygotes, which is hypothesized to occur because of local nonrandom mating. This is supported by values from Wrights F-statistics which indicated that F_{IS} (.184) is the largest contributor to the overall inbreeding coefficient. An increase in sample size for all populations and a detailed resampling of the Dancing Prairie population focusing on spatial, temporal and demographic variables is recommended. Additional genetic analyses of geographic differentiation are also proposed as a basis for future conservation and management decisions.

Introduction

Silene spaldingii is a rare perennial occurring on prairie remnants in Idaho, Oregon, Montana and Washington. Currently, research efforts are underway to gather the data necessary to successfully manage the long-term viability of this species. An important step in this process is understanding the genetic variability and structure within and among populations. This genetic information can provide important insights into areas such as reproductive biology, inbreeding, and ecotypic variation, and help establish protection priorities and guidelines for population restoration and seed transfer.

Sampling Procedures

Leaf samples of Silene spaldingii were collected by Montana Natural Heritage and BLM personnel from five study sites distributed across Idaho, Montana, Oregon, and Washington (Table 1). These can be grouped into a northern group (Lamona and Dancing Prairie) and a southern group (Garden Creek, Clear Lake Ridge and Kramer), with the latter being further subdivided into a Hells Canyon (Garden Creek and Clear Lake Ridge) group and a Palouse population (Kramer). Collections were made of green leaf material, using the freshest available intact leaf. A leaf was collected, sealed between damp paper towels in zip-loc plastic bags, stored on ice, and mailed in overnight mail to the University of Idaho. Sample sizes were extremely small, ranging from eight to twenty-five individuals, due to an extremely dry summer and poor growing conditions.

Table 1. Sample Locations of Silene spaldingii.

Garden Creek	(GC)	Hells Canyon, Idaho
Clear Lake Ridge	(CLR)	Hells Canyon, Oregon
Kramer	(KR)	Kramer, Washington
Lamona	(LM)	Lamona, Washington
Dancing Prairie	(DP)	Tobacco Plains, Montana

Electrophoretic Analysis

Electrophoretic protocols employed were primarily those described in Brunsfeld et al. (1991) and Soltis et al. (1983). Leaf tissue was ground in Tris-HCL grinding buffer, absorbed onto filter paper wicks, loaded into starch gels, and electrophoresed for approximately six hours. In preliminary tests seventeen enzymes were stained: aconitase (ACN), alcohol dehydrogenase (ADH), acid phosphatase (APH), aspartate aminotransferase (AAT), esterase (EST), glutamate dehydrogenase (GDH), hexokinase (HK), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), menadione reductase (MNR), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6-PGD), shikimate

dehydrogenase (SKDH), and triosephosphate isomerase (TPI). Of the enzymes tested, six (EST, LAP, 6-PGD, PGI, PGM, and TPI) produced enzyme activity and were analyzed in all subsequent gel runs.

The genetic basis of enzyme banding patterns was inferred from observed segregation patterns in light of typical subunit structure and subcellular compartmentalization (Gottlieb 1981, Weeden and Wendel 1989). For enzymes with more than one locus, the isozymes were numbered sequentially, with the most anodal isozyme designated 1. Allozymes were labeled alphabetically starting with the fastest allozyme. Enzyme data were analyzed using BIOSYS-1 (Swofford and Selander 1981) and GENESTAT (Lewis unpublished program). Three measures of genetic diversity were calculated: mean number of alleles per locus (A), percentage of loci polymorphic (P), and mean expected heterozygosity (H_e). Genetic divergence among populations and species was analyzed using Nei's (1978) unbiased genetic identity and distance measures calculated by BIOSYS-1, and a UPGMA cluster analysis of identity values was performed. Neighbor-joining was also conducted using Nei's unbiased distance values.

DNA Analysis

Total DNA was extracted from each leaf sample using a modified CTAB method following Brunsfeld et al. 1992. Purified DNA was next quantified and diluted to a uniform concentration (10 ng/ul). A known portion of the plant chloroplast genome, Open Reading Frame (ORF) 178 located within the small single copy, was amplified using the polymerase chain reaction (PCR). In PCR, a heat-stable enzyme acts as a molecular xerox copier which repeatedly replicates the same segment of DNA. This product was then digested using twelve different restriction endonucleases, which recognize specific sequences of DNA and cleave the strands at that location. The restriction fragments were then loaded into a acrylamide gel, electrophoresed for approximately three hours and visualized using silver staining (Promega, 1994).

Results

Enzyme Electrophoresis

Ten loci (EST, LAP, 6PGD-1, 6PGD-2, PGI-1, PGI-2, PGM-1, PGM-2, TPI-1, and TPI-2) were resolved for 96 individuals from five populations. Four of the loci (EST, PGI-1, PGI-2, PGM-1, and PGM-2) exhibited polymorphism. Measures of genetic variation of these five populations are given in Table 2. Mean sample size per locus varies between 8.8 (Kramer) and 24.2 (Lamona) individuals. The mean number of alleles per locus ranged from 1.4 in the Lamona population to 1.8 in the Clear Lake Ridge population. The number of loci exhibiting polymorphism ranged between 3 (30%) and 4 (40%). There appears to be no correlation

between the number of individuals in a sample and the mean number of alleles per locus or the percentage of loci which are polymorphic. The mean heterozygosity for all loci ranged from .198 in the Clear Lake Ridge population to .052 in the Dancing Prairie population. In three of the populations, Garden Creek, Dancing Prairie and Lamona, the observed heterozygosity was lower than values based upon Hardy-Weinberg expectations, while the Clear Lake Ridge and Kramer populations closely match expected values. A Chi-squared test for deviation from Hardy-Weinberg equilibrium was performed for each polymorphic locus in each population, and significant deviations were found for two of the four loci in the Dancing Prairie population ($P<.007$). An excess of homozygotes, based upon Hardy-Weinberg expectations, was found in all populations except Kramer (Table 3).

Genetic structure within and among populations was analyzed using Wright's F-statistics and values are given in Table 4. Following Wright (1965), F_{IT} and F_{IS} are the correlations of uniting gametes within an individual relative to those drawn at random from the total population and subpopulations respectively. Among population diversity F_{ST}, is the correlation of uniting gametes drawn at random from each subpopulation. Put simply, each F statistic is a measure of fixation, or reduction of heterozygosity due to nonrandom mating. For example, F_{IT} is the overall reduction of heterozygosity of an individual relative to the total population due to nonrandom mating. Whereas F_{IS} is the reduction in heterozygosity due to nonrandom mating within subpopulations and F_{ST} is the reduction in heterozygosity of a subpopulation due to processes such as random genetic drift (Hartl and Clark, 1989). Mean F statistics for Silene spaldingii are $F_{IT}=.258$; $F_{IS}=.184$; $F_{ST}=.091$.

Pairwise genetic distances between populations were calculated over all loci using Nei's (1978) unbiased genetic distance, which is given in Table 5. Unbiased distances, range from 0.00 between Clear Lake Ridge and Garden Creek to .040 between Lamona and Garden Creek. A dendrogram using a UPGMA cluster analysis based on Nei's (1978) unbiased genetic identity values is presented in Figure 1, grouping the northern populations, Lamona and Dancing Prairie, and the southern populations, Garden Creek, Clear Lake Ridge and Kramer. Neighbor-joining (Lewis, unpublished program) was also conducted and the resulting topology, which is congruent with the UPGMA, clearly shows a northern grouping and a southern grouping of the populations (Figure 2).

Restriction Site Analysis

The digest of the ORF 178 from the small single copy (SSC) region of the $_{cp}$DNA revealed differences that are currently being further explored.

Discussion

Preliminary data obtained for Silene spaldingii are comparable to that reported for other rare Silene species. Four of the loci scored were polymorphic as compared to the five in Silene regia, another rare plant occurring on prairie remnants in the midwest (Doland, 1994). In Silene hawaiiensis a total of twenty-one loci were scored with ten of those being polymorphic, as opposed to our total of ten loci scored with four being polymorphic. Yet, the genetic distance values (Nei, 1978) for Silene spaldingii are greater between northern Washington and Idaho than the distance values between populations on Maui and the Big Island, both of which are known for extremely high occurrences of endemism (Westerbergh and Saura, 1994).

Mean values of A, P, and H (Table 2) for Silene spaldingii reveal that at the species level, the electrophoretic data are generally consistent with the hypothesis of an outcrossing, animal (insect) pollinated breeding system (Hamrick and Godt, 1989). Structuring of genetic diversity within populations (F_{IS}=.184) is reflected in an excess of homozygotes. This may be due to occasional self-fertilization which has been demonstrated to be possible in this species (Lesica, 1993). The low value of F_{ST} (.091) indicates that genetic differentiation associated with population subdivision is a minor component of the genetic structuring. This is inconsistent with the expectations of current population genetics theory, which predicts that subdivision (F_{ST}) would be high when populations are small and migration of gametes between populations is low due to geographic isolation, conditions currently observed in Silene spaldingii. The inconsistency of the (F_{ST}) value is most easily explained by the hypothesis that the geographic distribution was much more extensive than is currently observed, and that drift is observed to be negligible because of a long generation time.

The idea that Silene spaldingii is primarily outcrossing is supported, for the most part, by the Chi-squared test for deviation from Hardy-Weinberg equilibrium. If outcrossing is the primary breeding system, then random mating in populations would be expected, and the frequencies of homozygotes and heterozygotes should be close to Hardy-Weinberg equilibrium. All of the populations except Dancing Prairie were close to Hardy-Weinberg equilibrium. Hypotheses to explain the significant deficiency of heterozygotes at Dancing Prairie include: 1) selection, 2) higher than expected selfing due to lack of pollinators or poor synchronization between pollinators and plants, and 3) nonrandom mating due to localized spatial and/or temporal structuring.

Under the selection hypothesis, heterozygotes are at a selective disadvantage compared to homozygotes. This hypothesis does not seem likely in light of work by Lesica (1993), which

suggested that fitness (and presumably heterozygosity) was conferred though outcrossing. However, selection by environmental factors such as fire, drought and disease have yet to be examined. Sampling of this population occurred during the latter part of the growing season, and may reflect selection pressures favoring homozygous individuals.

In the "higher than expected selfing" hypothesis, an increase in homozygotes results from a lack of pollinators. Selfing, which presumably increases homozygosity over time, may be increased because floral development occurs before pollinators, such as Bombus nevadensis, are present. Given that seed set normally occurs during the driest portion of the year, an advantage may accrue to the seeds which begin development early in the season. A related hypothesis is that pollinators may be absent from the site for several years, resulting in cohorts predominately composed of homozygotes derived from selfing. This could be easily tested by evaluating heterozygosity in different demographic classes. Another hypothesis is that the pollinator observed at Dancing Prairie is different from the other populations, promoting a higher incidence of selfing.

One of the more plausible hypotheses is that excess homozygosity is a result of predominant mating between sibs and/or offspring. This can arise through spatial and/or temporal aggregation of close relatives. Subpopulations may be separated physically by distance, or temporally by existing in microsites that promote different phenological development. This hypothesis is supported by the high F_{IS} value obtained (.184), which suggests the total reduction in heterozygosity due to nonrandom mating within subpopulations is the largest contributor to the overall inbreeding coefficient.

Future Research

Preliminary genetic work to date suggests the need for additional research, including:

1) Conduct a detailed resampling and analysis of the Dancing Prairie population. Research into potentially important spatial, temporal or demographically-based genetic structure should be incorporated into ongoing conservation biology studies.

2) Attempt to increase sample size for all populations where sampling was limited by drought conditions in 1993.

3) Confirm regional genetic differences within Silene spaldingii suggested by preliminary DNA analyses.

Table 2. Summary of mean number of alleles, percentage polymorphic loci and mean heterozygosity by population (standard errors in parentheses).

Population	Mean sample size per Locus	A Mean no. of alleles per locus	P Percentage of loci polymorphic	H Mean heterozygosity Direct-count	HdyWbg expected*
1. GARDEN CREEK, ID	12.9 (1.4)	1.7 (0.3)	40.0	.169 (.072)	.212 (.090)
2. CLEAR LAKE, OR	17.8 (1.4)	1.8 (0.4)	40.0	.198 (.085)	.194 (.087)
3. KRAMER, WA	8.8 (0.1)	1.6 (0.3)	30.0	.146 (.077)	.148 (.081)
4. DANCING PRAIRIE, MT	15.1 (0.1)	1.8 (0.3)	40.0	.052 (.024)	.164 (.088)
5. LAMONA, WA	24.2 (0.7)	1.4 (0.2)	30.0	.073 (.046)	.096 (.064)

* Unbiased estimate (see Nei, 1978)

Table 3. Coefficients for heterozygote deficiency or excess and a summary of Chi-square test for deviation from Hardy-Weinberg equilibrium using Levene (1949) correction for small sample size employed in chi-square analyses.

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D	Chi-square	DF	P
GARDEN CREEK, ID (GC)							
EST-1	12	14.256	.137	-.158	4.307	6	.635
PGI-1	5	6.185	.162	-.192	3.323	3	.344
PGI-2	5	6.517	.206	-.233	.896	1	.344
PGM-2	2	2.667	.167	-.250	.400	1	.527
CLEAR LAKE RIDGE, OR (CLR)							
EST-1	15	18.725	.183	-.199	8.559	6	.200
PGI-1	8	7.343	-.121	.089	1.786	3	.618
PGI-2	7	6.023	-.189	.162	.662	1	.416
PGM-2	7	5.952	-.232	.176	1.058	3	.787
KRAMER, WA (KR)							
EST-1	5	5.533	.036	-.096	7.821	6	.251
PGI-1	4	3.933	-.085	.017	3.182	3	.364
PGM-2	3	2.647	-.200	.133	.229	1	.633
DANCING PRAIRIE, MT (DP)							
EST-1	2	11.774	.825	-.830	40.345	6	.000
PGI-1	2	8.034	.742	-.751	12.191	3	.007
PGI-2	1	1.000	-.034	.000	.000	1	1.000
PGM-2	3	4.517	.313	-.336	9.070	3	.028
LAMONA, WA (LM)							
EST-1	8	11.286	.271	-.291	4.091	3	.252
PGI-1	5	6.106	.164	-.181	.903	1	.342
PGM-2	2	1.959	-.042	.021	.021	1	.884

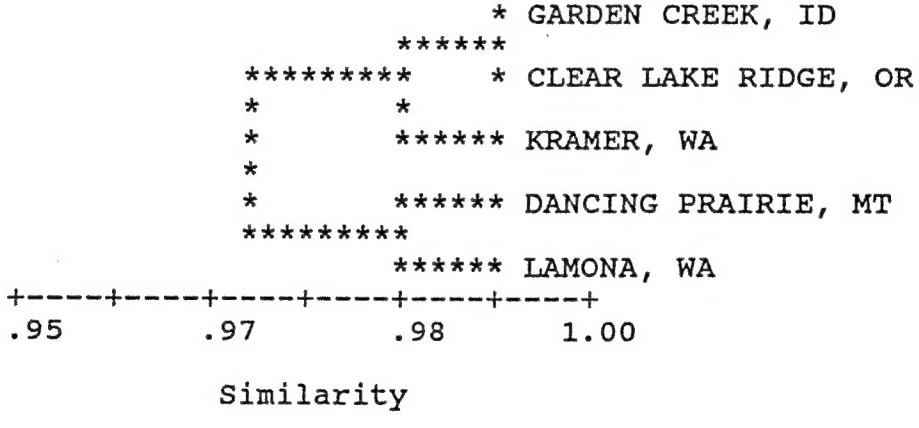
Table 4. Genetic differentiation of populations by F-statistics (Wright, 1965) over all loci.

Locus	F(IS)	F(IT)	F(ST)
EST-1	.297	.361	.091
PGI-1	.199	.229	.039
PGI-2	.045	.191	.153
PGM-2	-.005	.117	.121
Mean	.184	.258	.091

Table 5. Nei (1978) unbiased genetic distance

Garden Creek, ID	.000				
Clear Lake Ridge, OR	.000	.000			
Kramer, WA	.008	.009	.000		
Dancing Prairie, MT	.018	.011	.009	.000	
Lamona, WA	.040	.027	.024	.008	.000

Figure 1. Cluster analysis using UPGMA of Nei's (1978) unbiased genetic identity.



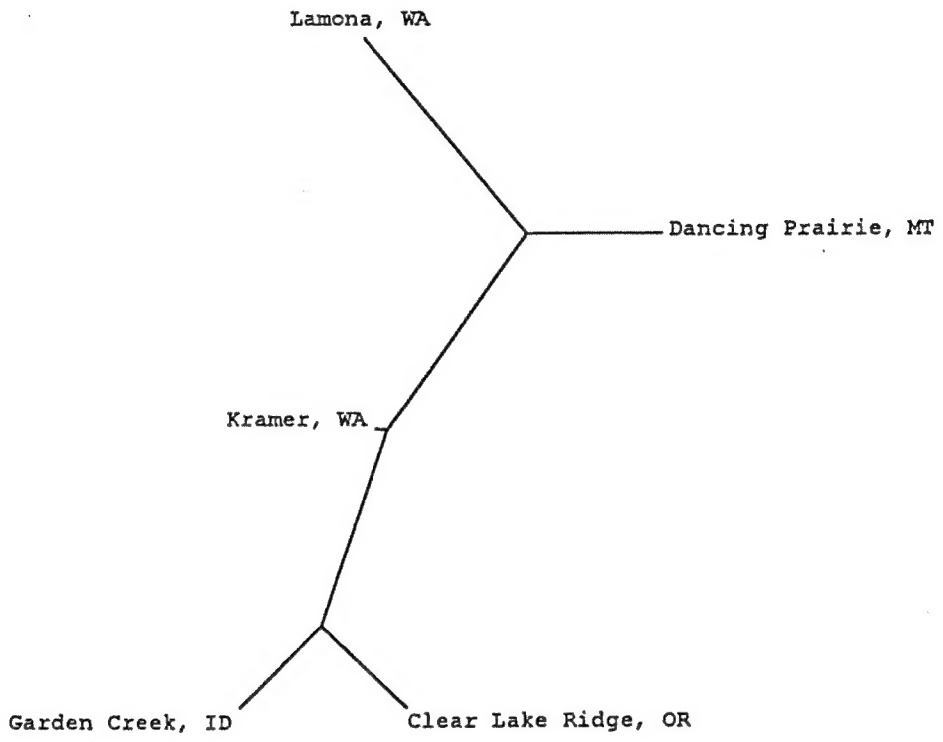


Figure 2. Neighbor-Joining using Nei's (1978) unbiased genetic distance.

Literature Cited

Brunsfeld, S.J., D.E. Soltis, and P.S. Soltis. 1991. Patterns of genetic variation in Salix sect. Longifoliae (Salicaceae). American Journal of Botany 78:855-869.

Dolan, R.W. 1994. Patterns of isozyme variation in relation to population size, isolation, and phytogeographic history in royal catchfly (Silene regia:Caryophyllaceae). Amer. J. Bot. 81(8):965-971.

Gottlieb, L.D. 1981. Electrophoretic evidence and plant populations. Progress in Phytochemistry 7:1-45.

Hamrick, J.L. and M.J.W. Godt. 1989. Allozyme diversity in plant species. *In* A.H.D. Brown, M.T. Clegg, A.L. Kahler, and S.B. Weir [eds.], Plant population genetics, breeding, and genetic resources, 43-63. Sinauer, Sunderland, MA.

Hartl, D.L. and A. G. Clark. 1989. Principles of Population Genetics. Sinauer, Sunderland, MA.

Lesica, P. 1993. Loss of fitness resulting from pollinator exclusion in Silene spaldingii (Caryophyllaceae). Madrono 40(4):193-201.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583-590.

Soltis, D.E., C. Haufler, D. Darrow, and G.J. Gastony. 1983. Starch gel electrophoresis of ferns. Am. Fern J. 73: 9-27.

Swofford, D.L. and R.B. Selander. 1981. BIOSYS-1. University of Illinois, Urbana.

Weeden, N.F., and J.F. Wendel. 1989. Genetics of plant isozymes. *In* D.E. Soltis and P.S. Soltis [eds.], Isozymes in plant biology, 46-72. Dioscorides Press, Portland, OR.

Westerbergh, A. and A. Saura. 1994. Genetic differentiation in endemic Silene (Caryophyllaceae) on the Hawaiian islands. Amer. J. Bot. 81(11):1487-1493.

Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19:395-420.

Appendix I. Allele frequencies of populations analyzed.

	Population				
Locus	GC	CLR	KR	DP	LM
EST-1					
(N)	20	26	8	16	18
A	.150	.231	.125	.250	.250
B	.200	.288	.063	.406	.528
C	.450	.385	.438	.188	.000
D	.200	.096	.375	.156	.222
TPI-1					
(N)	14	18	9	15	25
A	1.000	1.000	1.000	1.000	1.000
TPI-2					
(N)	14	18	9	15	25
A	1.000	1.000	1.000	1.000	1.000
LAP-1					
(N)	14	18	9	15	25
A	1.000	1.000	1.000	1.000	1.000
PGD-1					
(N)	14	18	9	15	25
A	1.000	1.000	1.000	1.000	1.000
PGD-2					
(N)	14	18	9	15	25
A	1.000	1.000	1.000	1.000	1.000
PGI-1					
(N)	14	18	8	15	24
A	.714	.750	.688	.567	.854
B	.250	.194	.250	.400	.146
C	.036	.056	.063	.033	.000
PGI-2					
(N)	15	22	9	15	25
A	.300	.159	.000	.033	.000
B	.700	.841	1.000	.967	1.000
PGM-1					
(N)	5	11	9	15	25
A	1.000	1.000	1.000	1.000	1.000
PGM-2					
(N)	5	11	9	15	25
A	.000	.045	.000	.066	.040
B	.600	.591	.833	.833	.960
C	.400	.364	.167	.100	.000

GC - Garden Creek, Idaho
CLR - Clear Lake Ridge, Oregon
KR - Kramer, Washington
DP - Dancing Prairie, Montana
LM - Lamona, Washington

Appendix II. Allele frequencies and genetic variability measures by population.

Population: GARDEN CREEK, ID (GC)

	Locus and sample size								
Allele	EST-1 20	TPI-1 14	TPI-2 14	LAP-1 14	PGD-1 14	PGD-2 14	PGI-1 14	PGI-2 15	PGM-1 5
A	.150	1.000	1.000	.000	1.000	1.000	.714	.300	1.000
B	.200	.000	.000	1.000	.000	.000	.250	.700	.000
C	.450	.000	.000	.000	.000	.000	.036	.000	.000
D	.200	.000	.000	.000	.000	.000	.000	.000	.000
H	.695	.000	.000	.000	.000	.000	.426	.420	.000
H(unb)	.713	.000	.000	.000	.000	.000	.442	.434	.000
H(D.C.)	.600	.000	.000	.000	.000	.000	.357	.333	.000

Allele	PGM-2 5
A	.000
B	.600
C	.400
D	.000
H	.480
H(unb)	.533
H(D.C.)	.400

Mean heterozygosity per locus (biased estimate) = .202 (S.E. .086)
Mean heterozygosity per locus (unbiased estimate) = .212 (S.E. .090)
Mean heterozygosity per locus (direct-count estimate) = .169 (S.E. .072)

Mean number of alleles per locus = 1.70 (S.E. .33)

Percentage of loci polymorphic (0.95 criterion) = 40.00

Population: CLEAR LAKE RIDGE, OR (CLR)

	Locus and sample size								
Allele	EST-1 26	TPI-1 18	TPI-2 18	LAP-1 18	PGD-1 18	PGD-2 18	PGI-1 18	PGI-2 22	PGM-1 11
A	.231	1.000	1.000	.000	1.000	1.000	.750	.159	1.000
B	.288	.000	.000	1.000	.000	.000	.194	.841	.000
C	.385	.000	.000	.000	.000	.000	.056	.000	.000
D	.096	.000	.000	.000	.000	.000	.000	.000	.000
H	.706	.000	.000	.000	.000	.000	.397	.268	.000
H(unb)	.720	.000	.000	.000	.000	.000	.408	.274	.000
H(D.C.)	.577	.000	.000	.000	.000	.000	.444	.318	.000

Allele	PGM-2 11
A	.045
B	.591
C	.364
D	.000
H	.517
H(unb)	.541
H(D.C.)	.636

Mean heterozygosity per locus (biased estimate) = .189 (S.E. .084)
Mean heterozygosity per locus (unbiased estimate) = .194 (S.E. .087)
Mean heterozygosity per locus (direct-count estimate) = .198 (S.E. .085)

Mean number of alleles per locus = 1.80 (S.E. .36)

Percentage of loci polymorphic (0.95 criterion) = 40.00

Population: KRAMER, WA (KR)

	Locus and sample size								
Allele	EST-1 8	TPI-1 9	TPI-2 9	LAP-1 9	PGD-1 9	PGD-2 9	PGI-1 8	PGI-2 9	PGM-1 9
A	.125	1.000	1.000	.000	1.000	1.000	.688	.000	1.000
B	.063	.000	.000	1.000	.000	.000	.250	1.000	.000
C	.438	.000	.000	.000	.000	.000	.063	.000	.000
D	.375	.000	.000	.000	.000	.000	.000	.000	.000
H	.648	.000	.000	.000	.000	.000	.461	.000	.000
H(unb)	.692	.000	.000	.000	.000	.000	.492	.000	.000
H(D.C.)	.625	.000	.000	.000	.000	.000	.500	.000	.000

Allele	PGM-2 9
A	.000
B	.833
C	.167
D	.000
H	.278
H(unb)	.294
H(D.C.)	.333

Mean heterozygosity per locus (biased estimate) = .139 (S.E. .076)
Mean heterozygosity per locus (unbiased estimate) = .148 (S.E. .081)
Mean heterozygosity per locus (direct-count estimate) = .146 (S.E. .077)

Mean number of alleles per locus = 1.60 (S.E. .34)

Percentage of loci polymorphic (0.95 criterion) = 30.00

Population: DANCING Prairie, MT (DP)

	Locus and sample size								
Allele	EST-1 16	TPI-1 15	TPI-2 15	LAP-1 15	PGD-1 15	PGD-2 15	PGI-1 15	PGI-2 15	PGM-1 15
A	.250	1.000	1.000	.000	1.000	1.000	.567	.033	1.000
B	.406	.000	.000	1.000	.000	.000	.400	.967	.000
C	.188	.000	.000	.000	.000	.000	.033	.000	.000
D	.156	.000	.000	.000	.000	.000	.000	.000	.000
H	.713	.000	.000	.000	.000	.000	.518	.064	.000
H(unb)	.736	.000	.000	.000	.000	.000	.536	.067	.000
H(D.C.)	.125	.000	.000	.000	.000	.000	.133	.067	.000

Allele	PGM-2 15
A	.066
B	.833
C	.100
D	.000
H	.402
H(unb)	.416
H(D.C.)	.200

Mean heterozygosity per locus (biased estimate) = .159 (S.E. .085)
Mean heterozygosity per locus (unbiased estimate) = .164 (S.E. .088)
Mean heterozygosity per locus (direct-count estimate) = .052 (S.E. .024)

Mean number of alleles per locus = 1.80 (S.E. .33)

Percentage of loci polymorphic (0.95 criterion) = 30.00

Population: LAMONA, WA (LM)

	Locus and sample size								
Allele	EST-1 18	TPI-1 25	TPI-2 25	LAP-1 25	PGD-1 25	PGD-2 25	PGI-1 24	PGI-2 25	PGM-1 25
A	.250	1.000	1.000	.000	1.000	1.000	.854	.000	1.000
B	.528	.000	.000	1.000	.000	.000	.146	1.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.222	.000	.000	.000	.000	.000	.000	.000	.000
H	.610	.000	.000	.000	.000	.000	.249	.000	.000
H(unb)	.627	.000	.000	.000	.000	.000	.254	.000	.000
H(D.C.)	.444	.000	.000	.000	.000	.000	.208	.000	.000

Allele	PGM-2 25
A	.040
B	.960
C	.000
D	.000
H	.077
H(unb)	.078
H(D.C.)	.080

Mean heterozygosity per locus (biased estimate) = .094 (S.E. .063)
Mean heterozygosity per locus (unbiased estimate) = .096 (S.E. .064)
Mean heterozygosity per locus (direct-count estimate) = .073 (S.E. .046)

Mean number of alleles per locus = 1.40 (S.E. .22)

Percentage of loci polymorphic (0.95 criterion) = 20.00

Appendix III. Chi-square test for deviation from Hardy-Weinberg equilibrium using Levene (1949) correction for small sample size employed in chi-square analyses.

Population: GARDEN CREEK, ID (GC)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1						
	A-A	1	.385			
	A-B	0	1.231			
	A-C	2	2.769			
	A-D	2	1.231			
	B-B	1	.718			
	B-C	4	3.692			
	B-D	2	1.641			
	C-C	5	3.923			
	C-D	2	3.692			
	D-D	1	.718			
				4.307	6	.635
PGI-1						
	A-A	8	7.037			
	A-B	4	5.185			
	A-C	0	.741			
	B-B	1	.778			
	B-C	1	.259			
	C-C	0	.000			
				3.323	3	.344
PGI-2						
	A-A	2	1.241			
	A-B	5	6.517			
	B-B	8	7.241			
				.896	1	.344
PGM-2						
	B-B	2	1.667			
	B-C	2	2.667			
	C-C	1	.667			
				.400	1	.527

Chi-square test with pooling

Population: GARDEN CREEK, ID (GC)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1	Homozygotes for most common allele	5	3.923			
	Common/rare heterozygotes	8	10.154			
	Rare homozygotes and other heterozygotes	7	5.923	.948	1	.330
PGI-1	Homozygotes for most common allele	8	7.037			
	Common/rare heterozygotes	4	5.926			
	Rare homozygotes and other heterozygotes	2	1.037	1.652	1	.199

Population: CLEAR LAKE RIDGE, OR (CLR)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1						
	A-A	2	1.294			
	A-B	4	3.529			
	A-C	3	4.706			
	A-D	1	1.176			
	B-B	4	2.059			
	B-C	3	5.882			
	B-D	0	1.471			
	C-C	5	3.725			
	C-D	4	1.961			
	D-D	0	.196			
				8.559	6	.200
PGI-1						
	A-A	10	10.029			
	A-B	6	5.400			
	A-C	1	1.543			
	B-B	0	.600			
	B-C	1	.400			
	C-C	0	.029			
				1.786	3	.618
PGI-2						
	A-A	0	.488			
	A-B	7	6.023			
	B-B	15	15.488			
				.662	1	.416
PGM-2						
	A-A	0	.000			
	A-B	1	.619			
	A-C	0	.381			
	B-B	3	3.714			
	B-C	6	4.952			
	C-C	1	1.333			
				1.058	3	.787

CLEAR LAKE RIDGE, OR (CLR)

Chi-square test with pooling

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1	Homozygotes for most common allele	5	3.725			
	Common/rare heterozygotes	10	12.549			
	Rare homozygotes and other heterozygotes	11	9.725	1.121	1	.290
PGI-1	Homozygotes for most common allele	10	10.029			
	Common/rare heterozygctes	7	6.943			
	Rare homozygotes and other heterozygotes	1	1.029	.001	1	.971
PGM-2	Homozygotes for most common allele	3	3.714			
	Common/rare heterozygotes	7	5.571			
	Rare homozygotes and other heterozygotes	1	1.714	.801	1	.371

Population: KRAMER, WA (KR)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1						
	A-A	0	.067			
	A-B	1	.133			
	A-C	0	.933			
	A-D	1	.800			
	B-B	0	.000			
	B-C	0	.467			
	B-D	0	.400			
	C-C	2	1.400			
	C-D	3	2.800			
	D-D	1	1.000			
				7.821	6	.251
PGI-1						
	A-A	4	3.667			
	A-B	3	2.933			
	A-C	0	.733			
	B-B	0	.400			
	B-C	1	.267			
	C-C	0	.000			
				3.182	3	.364
PGM-2						
	B-B	6	6.176			
	B-C	3	2.647			
	C-C	0	.176			
				.229	1	.633

Chi-square test with pooling

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1	Homozygotes for most common allele	2	1.400			
	Common/rare heterozygotes	3	4.200			
	Rare homozygotes and other heterozygotes	3	2.400	.750	1	.386
PGI-1	Homozygotes for most common allele	4	3.667			
	Common/rare heterozygotes	3	3.667			
	Rare homozygotes and other heterozygotes	1	.667	.318	1	.573

Population: DANCING PRAIRIE, MT (DP)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1						
	A-A	3	.903			
	A-B	1	3.355			
	A-C	0	1.548			
	A-D	1	1.290			
	B-B	6	2.516			
	B-C	0	2.516			
	B-D	0	2.097			
	C-C	3	.484			
	C-D	0	.968			
	D-D	2	.323			
				40.345	6	.000
PGI-1						
	A-A	8	4.690			
	A-B	1	7.034			
	A-C	0	.586			
	B-B	5	2.276			
	B-C	1	.414			
	C-C	0	.000			
				12.191	3	.007
PGI-2						
	A-A	0	.000			
	A-B	1	1.000			
	B-B	14	14.000			
				.000	1	1.000
PGM-2						
	A-A	0	.034			
	A-B	2	1.724			
	A-C	0	.207			
	B-B	11	10.301			
	B-C	1	2.586			
	C-C	1	.103			
				9.670	3	.028

Chi-square test with pooling

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P
EST-1	Homozygotes for most common allele	6	2.516			
	Common/rare heterozygotes	1	7.968			
	Rare homozygotes and other heterozygotes	9	5.516	13.117	1	.000
PGI-1	Homozygotes for most common allele	8	4.690			
	Common/rare heterozygotes	1	7.621			
	Rare homozygotes and other heterozygotes	6	2.690	12.163	1	.000
PGM-2	Homozygotes for most common allele	11	10.345			
	Common/rare heterozygotes	3	4.310			
	Rare homozygotes and other heterozygotes	1	0.345	1.685	1	.194